PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
C12N 5/00	A1	WO 93/00423
		(43) International Publication Date: 7 January 1993 (07.01.93)

(21) International Application Number: PCT/DK92/00190

(22) International Filing Date: 18 June 1992 (18.06.92)

(30) Priority data:
91610054.8 21 June 1991 (21.06.91) EP
(34) Countries for which the regional or international application was filed: DK et al.

(71) Applicant *(for all designated States except US):* NOVO NORDISK A/S [DK/DK]; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK).

(72) Inventor; and
(75) Inventor/Applicant *(for US only) :* SUHR-JESSEN, Peter, Bernt [DK/DK]; Mosegård Park 25, DK-3500 Værløse (DK).

(74) Common Representative: NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: IRON CHELATE CULTURE MEDIUM ADDITIVE

(57) Abstract

A culture medium additive comprises an iron chelate of a soluble iron salt and an alkali metal or alkaline earth metal citrate. The additive is a suitable iron source for serum-free or protein-free culture media.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

Iron chelate culture medium additive.

FIELD OF INVENTION

The present invention relates to an iron supplement for culture media, primarily serum-free or protein-free media, for growing mammalian cells, and a culture medium containing said iron supplement.

BACKGROUND OF THE INVENTION

Until fairly recently, conventional media for growing mammalian cells contained serum as an important source of growth factors in the requisite concentrations for the growth and natural multiplication of the cells. The presence of serum or specific added proteins in culture media, however, suffers from the disadvantage that the purification of the desired protein product from the mammalian culture is made more difficult and that there is an increased risk of contamination by infectious agents. It is therefore an important aim in the field of mammalian cell culture to develop media in which the components in serum necessary for cell growth have been replaced with non-proteinaceous substances serving the same purpose. Serum-free or protein-free media have therefore become increasingly important for the cultivation of mammalian cells in the production of biological materials (e.g. monoclonal antibodies, natural or recombinant pharmaceuticals, or the like).

Most serum-free media are based on a commercially available basal medium (e.g. MEM, Ham, RPMI) supplemented with insulin, transferrin, selenium, growth factors, and some protein and lipid sources [Hamilton et al., In Vitro 13: 537-547, 1977; Ham et al., Methods Enzymol. 58: 44-93, 1979; Maciag et al., Cell Biol. Int. Rep. 4: 43-50, 1980; Barnes, BioTechnology 5: 534-540, 1987; Fiorentini et al., Am. Biotech. Lab. 8: 35-37, 1990; Bjare, J. Biotech. 15: 147-154, 1990; Hewlett, Cytotechnology 5: 3-14, 1991].

SUMMARY OF THE INVENTION

It has now been found possible to replace transferrin as the iron source in serum-free media by a non-protein chelate of citrate and an iron salt.

Accordingly, the present invention relates to a culture medium additive comprising an iron chelate of a soluble iron salt and an alkali metal or alkaline earth metal citrate. Iron chelates for serum-free media have previously been proposed, e.g. in EP 274 445 describing a culture medium additive containing an iron-EDTA/citric acid chelate and aurin tricarboxylic acid. The iron chelate additive of the present invention has the advantage over the one proposed in EP 274 445 that it is composed of inexpensive constituents, and that it contains fewer constituents which might be a source of contamination.

In another aspect, the present invention relates to a culture medium for growing mammalian cells, the medium comprising an iron chelate of a soluble iron salt and an alkali metal or alkaline earth metal citrate.

DETAILED DISCLOSURE OF THE INVENTION

To avoid iron precipitation and potential toxic effects of the iron on the cultured cells, the citrate chelator should be mixed with the iron salt so as to generate an equilibrium prior to the addition to the culture medium. This equilibrium may for instance be formed in a concentrated stock solution and, and the process speeded up by stirring, autoclaving, etc. In the preparation of the iron additive, the requisite equilibrium is most conveniently reached when the alkali metal or alkaline earth metal citrate is present in a molar excess relative to the iron salt, in particular a ratio of the citrate to the iron salt of more than 1:1 and less than 500:1.

Suitable iron salts for inclusion in the additive of the invention may be selected from the group consisting of $FeCl_2$, $FeCl_3$, $FeSO_4$, $Fe_3(PO_4)_2$, $Fe(NO_3)_3$ and FeI_2. Examples of suitable alkali metal or alkaline earth metal citrates for inclusion in the additive of the invention are Na-citrate, K-citrate or Mg-citrate. In a particularly preferred embodiment, the iron salt included in the additive is $FeCl_2$ or $FeCl_3$, and the citrate is Na-citrate. In this case, a preferred molar ratio of Na-citrate to $FeCl_2/FeCl_3$ is between 2:1 and 200:1.

The culture medium in which the additive is intended to be included is preferably a medium for growing mammalian cells, the additive of the invention constituting an inexpensive iron source which mammalian cells have surprisingly been able to utilise. Thus, the medium may for instance be a low-serum medium or, preferably, a serum-free or protein-free medium in which it is important to provide a non-protein iron supplement. Although it has previously been described that the freshwater ciliate Tetrahymena thermophila is able to utilise pre-chelated iron citrate as the only iron source (cf. P.B. Suhr-Jessen and L. Rasmussen, Exp. Cell Res. 139, 1982, pp. 457-460; L. Rasmussen et al., J. Cell. Phys. 122, 1985, pp. 155-158), it has not been suggested that mammalian cells may also utilise a citrate/iron chloride chelate as the iron source in serum-free media. Biologically speaking, it is quite surprising that mammalian cells which exist in an environment enriched in nutrient components and under conditions of considerable osmotic pressure are able to assimilate nutrients in a similar way as a primitive freshwater organism specialized in surviving in a nutrient-poor environment.

The invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

EXAMPLE 1. BHK cells

Adherent BHK cells cultivated in coated T-flasks containing a serum-free nutrient medium for BHK cells (as described by Maciag et al. 1980, ibid) with transferrin as the only iron source (SFNMT), were concomitantly inoculated into a series of coated T-flasks containing serum-free nutrient medium lacking transferrin (SFNM-) but supplemented with a chelated stock solution of Na-citrate and iron chloride. Experiments 1 to 3 had different durations and the experimental citrate concentration was 2 mM, 2 mM, and 5 mM (final conc.), respectively. Parallel control cultures were cultivated in SFNMT.

Each cell culture was independently treated with respect to replacement of used medium with fresh serum-free medium of the identical kind or sub-cultivation into new T-flask containing fresh serum-free medium of the identical kind. At the end of the experiment, the total number of doublings in each medium was calculated:

EXAMPLE 1. BHK	EX. 1 2 mM citrate	EX. 2 2 mM Citrate	EX. 3 5 mM Citrate
final μM $FeCl_3$	cell doublings	cell doublings	cell doublings
0	< 2	3.9	< 1
3	< 2	n.d.	n.d.
10	< 2	n.d.	n.d.
30	< 3	n.d.	n.d.
100	13	5.3	14
300	8.5	5.5	13.4
500	n.d.	n.d.	14.4
1.000	8	1.7	15
SFNMT*	6	4.3	10.5

* Citrate and iron chloride was not added to SFNMT

EXAMPLE 2. BHK cells

BHK cells were inoculated into spinner flasks containing SFNM- for BHK cells (see example 1) supplemented with a chelated citrate-iron stock solution resulting in 2 mM Citrate and 100 μM $FeCl_3$ (final conc.). Following a few hours where cells were allowed to adhere to coated microcarriers, cells spread, propagated and remained essentially confluent and healthy for more than two weeks when the experiment was terminated.

EXAMPLE 3. CHO cells

Adherent CHO cells cultivated in coated T-flasks containing a serum-free nutrient medium for CHO cells (as described by Ham et al. 1979, ibid.) with transferrin as the only iron source (SFNMT), were concomitantly inoculated into a series of coated T-flasks containing serum-free nutrient medium lacking transferrin (SFNM-) but supplemented with a chelated stock solution of Na-citrate and iron chloride. Experiments 1 and 2 had different durations and the experimental citrate concentration was 2 mM (final conc.). Parallel control cultures were cultivated in SFNMT.

Each cell culture was independently treated with respect to replacement of used medium with fresh serum-free medium of the identical kind or sub-cultivation into new T-flask containing fresh serum-free medium of the identical kind.

At the end of the experiment, the total number of doublings in each medium was calculated:

EXAMPLE 3. CHO	EX. 1 2 mM citrate	EX. 2 2 mM Citrate
final μM $FeCl_3$	cell doublings	cell doublings
0	< 1	< 1
3	< 1	< 1
10	4.2	1.3
30	10.9	10.4
100	11.2	9.4
300	10.6	9.3
1.000	12.4	9.0
SFNMT*	6.7	4.4

* Citrate and iron chloride was not added to SFNMT

EXAMPLE 4. CHO cells

CHO cells were inoculated into two spinner flasks containing SFNM- for CHO cells (see example 3) supplemented with chelated citrate-iron chloride stock solutions resulting in 2 mM Citrate and 100 and 300 μM $FeCl_3$ (final conc.), respectively. After a few hours where cells were allowed to adhere to coated micro carriers, cells spread, propagated and remained essentially confluent and healthy for more than two weeks when the experiment was terminated.

EXAMPLE 5. MYELOMA cells

SP2/0 myeloma cells cultivated in suspension culture in T-flasks containing an RPMI based serum-free nutrient medium (Shacter 1989, TIBTECH, 7, 248-253) with transferrin as the only iron source (SFNMT), were concomittantly inoculated into a series of T-flasks containing serum-free nutrient medium

lacking transferrin (SFNM-) but supplemented with a chelated stock solution of Na-citrate and iron chloride.

Each cell culture was independently treated with respect to replacement of used medium with fresh serum-free medium of the identical kind or sub-cultivation into new T-flask containing fresh serum-free medium of the identical kind.
At the end of the experiment, the total number of doublings in each medium was calculated:

EXAMPLE 5. SP2/0	EX. 1 2 mM Citrate
final μM $FeCl_3$	cell doublings
0	1.6
30	9.4
100	10.0
300	10.4
1.000	9.3
SFMNT*	5.1

* Citrate and iron chloride was not added to SFMNT

EXAMPLE 6. HYBRIDOMA cells

SP2/0 based hybridoma cells cultivated in suspension culture in T-flasks containing an RPMI based serum-free nutrient medium for hybridoma cells (Shacter 1989, TIBTECH, 7, 248-253) with transferrin as the only iron source (SFNMT), were concomittantly inoculated into a series of T-flasks containing serum-free nutrient medium lacking transferrin (SFNM-) but supplemented with a chelated stock solution of Na-citrate and iron chloride.

Each cell culture was independently treated with respect to replacement of used medium with fresh serum-free medium of the identical kind or sub-cultivation into new T-flask containing fresh serum-free medium of the identical kind.
At the end of the experiment, the total number of doublings in each medium was calculated:

EXAMPLE 6. Hybridoma	Ex. 1 2mM Citrate
final μM $FeCl_3$	cell doublings
0	2.5
30	11.5
100	14.0
300	13.5
1.000	13.4
SFNMT*	15.7

* Citrate and iron chloride was not added to SFNMT

CLAIMS

1. An culture medium additive comprising an iron chelate of a soluble iron salt and an alkali metal or alkaline earth metal citrate.

2. An additive according to claim 1, wherein the alkali metal or alkaline earth metal citrate is present in a molar excess relative to the iron salt

3. An additive according to claim 1 or 2, wherein the iron salt is selected from the group consisting of $FeCl_2$, $FeCl_3$, $FeSO_4$, $Fe_3(PO_4)_2$, $Fe(NO_3)_3$ and FeI_2.

4. An additive according to any of claims 1-3, wherein the alkali metal or alkaline earth metal citrate is selected from the group consisting of Na-citrate, K-citrate and Mg-citrate.

5. An additive according to any of claims 1-4, wherein the molar ratio of alkali metal or alkaline earth metal citrate to iron salt is more than 1:1 and less than 500:1.

6. An additive according to any of claims 1-5, wherein the culture medium in which it is included is for growing mammalian cells.

7. An additive according to any of claims 1-6, wherein the culture medium in which it is included is a serum-free or protein-free medium.

8. An additive according to any of claims 1-7, wherein the iron salt is $FeCl_2$ or $FeCl_3$, and wherein the citrate is Na-citrate.

9. An additive according to claim 8, wherein the molar ratio of Na-citrate to $FeCl_2/FeCl_3$ is between 2:1 and 200:1.

10. A culture medium for growing mammalian cells, the medium

comprising an iron chelate of a soluble iron salt and an alkali metal or alkaline earth metal citrate.

11. A culture medium according to claim 10, wherein the alkali metal or alkaline earth metal citrate is present in a molar excess relative to the iron salt.

12. A culture medium according to claim 10 or 11, wherein the iron salt is selected from the group consisting of $FeCl_2$, $FeCl_3$, $FeSO_4$, $Fe_3(PO_4)_2$, $Fe(NO_3)_3$ and FeI_2.

13. A culture medium according to any of claims 10-12, wherein the alkali metal or alkaline earth metal citrate is selected from the group consisting of Na-citrate, K-citrate and Mg-citrate.

14. A culture medium according to any of claims 10-13, wherein the molar ratio of alkali metal or alkaline earth metal citrate to iron salt is more than 1:1 and less than 500:1.

15. A culture medium according to any of claims 10-14, which is a serum-free or protein-free medium.

16. A culture medium according to any of claims 10-15, wherein the iron salt is $FeCl_2$ or $FeCl_3$, and wherein the citrate is Na-citrate.

17. A culture medium according to claim 16, wherein the molar ratio of Na-citrate to $FeCl_2/FeCl_3$ is between 2:1 and 200:1.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 92/00190

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	EP, A2, 0274445 (MEDI-CULT A/S) 13 July 1988, see the whole document --	1-17
X	GB, A, 2196348 (CESKOSLOVENSKA AKADMIE VED) 27 April 1988, see in particular page 1, line 112 - page 2, line 13	1,6,7, 10,11, 15
Y	 --	1-17
Y	Dialog Information services, File 351, WPI, Dialog accession no. 008681836, WPI accession no. 91-185855/26, Loeffler-Inst: "Chemical absorption of ammonia in viral replication medium - by adding iron citrate which reacts to form mixed ligand complex, used in prodn. of antigens for vaccines", DD 286612, A, 910131, 9126 (Basic) --	1-17

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd September 1992	25 -09- 1992
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carl Olof Gustafsson

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Dialog Information Services, File 351, WPI, Dialog accession no. 009027456, WPI accession no. 92-154816/19, Tosoh corp: "complete synthetic medium - contains iron citrate, ethanolamine and linolic acid, oleic acid and/or taurine, does not contain protein, cell growth factor, hormone and steroid", JP 4091786, A, 920325, 9219 (Basic) --	1,6,7, 10,11, 15
X	Tibtech, Vol. 7, September 1989 E. Shacter: "Serum-free media for bulk culture of hybridoma cells and the preparation of monoclonal antibodies", see page 248 - page 253 see page 249, right column --	1,6,7, 10,11, 15
A	National Library of Medicine, Database Medline, accession no.89124403, Schneider Y.: "Optimisation of hybridoma cell growth and monoclonal antibody secretion in a chemically defined, serum- and protein-free culture medium", & J Immunol Methods 1989 Jan 6;116(1):65-77 --	1
X	National Library of Medicine, Database Medline, accession no. 88284722, Kov:a:r J.: "Growth-stimulating effect of ferric citrate on hybridoma cells: characterization and relation to transferrin function", & Hybridoma 1988 Jun; 7(3):255-63 --	1,6,7, 10,11, 15
X	Dialog Information Services, Database BIOSIS, File 5, Dialog accession no. 9045773, Biosis accession no. 93030773, Franek F.: "Hybridoma growth and monoclonal antibody production in iron-rich protein-free medium effect of nutrient concentration", & Cytotechnology 7 (1), 1991, 33-38 --	1,6,7, 10,11, 15
X	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 05755034, Medline accession no. 86056034, Reddel RR.: "Cell cycle effects of iron depletion on T-47D human breast cancer cells", Exp Cell Res, Dec 1985, 161 (2) p277-84 --	1,6,7, 10,11, 15

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Dialog Information Services, File 155, MEDLINE, Dialog accession no.06308060, Medline accession no. 87282060, Hershko C. et al.: "Modification of iron uptake and lipid peroxidation by hypoxia, ascorbic acid, and alpha-tocopherol in iron-loaded rat myocardial cell cultures", & J Lab Clin Med Sep 1987 110 (3) p355-61 --	1,6,7, 10,11, 15
A	Dialog Information Services, File 155, MEDLINE, Dialog accession no.02890092, Medline accession no. 76071092, Hill JH et al.: "Iron-induced enhancement of 67Ga uptake in a model human leukocyte culture system", & J Nucl Med Dec 1975, 16 (12) p1183-6 --	1,6,7, 10,11, 15
A	Patent Abstracts of Japan, Vol 12, No 209, C504, abstract of JP 63- 77801, publ 1988-01-13 Nippon Zenyaku Kogyo K.K. --	1
A	Patent Abstracts of Japan, Vol 12, No 398, C538, abstract of JP 63-141584, publ 1988-06-14 Chemo Sero Therapeut Res Inst --	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 92/00190

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 28/08/92 The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0274445	88-07-13	AU-B-	596491	90-05-03
		AU-D-	1011588	88-07-14
		JP-A-	63279786	88-11-16
		US-A-	5045454	91-09-03
		US-A-	5045467	91-09-03
GB-A- 2196348	88-04-27	DE-A-	3733453	88-04-14
		FR-A-	2604727	88-04-08